International Journal of Medicine and Pharmaceutical Sciences (IJMPS)
ISSN(P): 2250-0049; ISSN(E): 2321-0095
Vol. 4, Issue 2, Apr 2014, 65-84

RECOMBINANT PROTEINS OF *MYCOBACTERIUM TUBERCULOSIS* – THE POTENTIAL SERODIAGNOSTIC CANDIDATES FROM DELETED REGIONS

KALAMANI VELMURUGAN & REKHA GOVINDAN

Associate Professor, Department of Biotechnology, Aarupadai Veedu Institute of Technology, Vinayaka Missions University, Kancheepuram, Tamil Nadu, India

ABSTRACT

Tuberculosis is one of the leading causes of death which haunted the mankind since antiquity and still is a global epidemic growing with increased incidence, especially in India. Bacillus Calmette-Guerin (BCG) is the only available vaccine for tuberculosis currently in use. But this vaccine shows a varied success rate (0% to 80%) in preventing tuberculosis. Gene deletion was an important part of the attenuation process that led to the BCG vaccine. The genome of *M. tuberculosis* comprises 4,411,529 bp and contains around 4,000 genes. Comparative genomics has identified several genomic regions in *Mycobacterium tuberculosis* and *Mycobacterium bovis* that are deleted in all tested BCG strains. The Region of Deletion 1 region is the best characterized of the deleted regions. It has been long speculated and recently been confirmed that the 9455 bp region of deletion 1 of *Mycobacterium tuberculosis* contributes to bacterial virulence. The identification of RD1 and the subsequent demonstration of its role in the virulence of *Mycobacterium tuberculosis* have fueled the speculation that this region could be exploited for the development of a vaccine for TB. Immunological tests based on detecting the presence of antibodies in the sera of active TB patients against various antigens of *M. tuberculosis* are useful for sero-diagnosis of TB and offer simple, rapid and cost effective methods most suitable for poor and developing countries. Several serodiagnostic assays use an optimized set of recombinant antigens from RD1 region of *Mycobacterium tuberculosis.* This may help to develop a novel anti-tuberculosis vaccine with higher efficiency than BCG by genetic engineering.

KEYWORDS: BCG, Region of Deletions, Serodiagnosis, TB Vaccines

INTRODUCTION

Tuberculosis (TB) is the most frequent cause of mortality due to a single infectious disease, which claims more than two million lives worldwide every year. The disease is caused by infection with *Mycobacterium tuberculosis* and remains the most important fatal infection of human beings. When an individual inhales aerosolized fluid droplets containing viable *Mycobacterium tuberculosis*, the larger droplets with bacilli are generally trapped in the airways and cleared. However, the smaller droplets, containing one to two bacilli enter into the lung and are phagocytosed by the resident alveolar macrophages. At this primary site of infection, bacilli multiply intracellularly. It may become dormant indefinitely inside the host (latent infection), or cause disease (primary tuberculosis) soon or many years after the infection. In reactivation tuberculosis the bacilli proliferate and cause active disease in only 5 to 10 percent of infected persons (Desprez and Heim, 1990). About two weeks later, secondary sites are seeded by the transport of bacilli within phagocytes through lymphatic and blood vessels. Mean time, the development of immune response leads to microscopic tubercle

formation. These small subsets of population with active disease are sufficient to ensure the bacilli transmission and survival in the community. By this way, one third of the world population is latently infected with *M. tuberculosis* and at risk of disease reactivation (Small and Fujiwara 2001; Betts *et al*., 2002).

In 2010, there were 8.8 million incident cases of TB, 1.1 million deaths from TB among HIV-negative people and an additional 0.35 million deaths from HIV-associated TB (WHO, 2011). This alarming trend has been increased by the emergence of multi-drug-resistant (MDR) and Extensively Drug resistant (XDR) strains of *M. tuberculosis*, which further compounds the TB epidemic (Petrini and Hoffner, 1999;Sturum, 2006) The frequency of infection is even higher in individuals who have a deficiency in their immune response (HIV infected individuals, people treated with immune suppressors etc.). The incidence of this ancient disease remains high and is increasing in many parts of the world due to the unfortunate synergism with Human Immunodeficiency Virus (HIV) infection (Pasqualoto and Ferreira, 2001).

MULTI-DRUG RESISTANT TUBERCULOSIS

The incidence of MDR-TB strains are on the rise and currently 50 million people are infected with MDR strains of tuberculosis worldwide (WHO Report, 2000).Low compliance with chemotherapy has been implicated in the increasing numbers of *M. tuberculosis* strains resistant to at least two first line drugs (Multi drug resistant tuberculosis), an event that could effectively turn the disease uncontrollable worldwide. Many pathogenic bacteria possess resistance plasmids, which can effect a rapid MDR transition to drug susceptible wild type strains and might confer resistance to many antibacterial substances at once. This has never been observed in *M. tuberculosis*, but it is known that the resistant and multi-resistant phenotypes are caused by random chromosomal mutations such as nucleotide insertions, deletions or substitutions in different genes of this organism (Petrini and Hoffner, 1999).

EXTENSIVELY-DRUG RESISTANT TUBERCULOSIS

The Centre for Disease Control and Prevention (CDC,USA) defined a new class of MDR, which was named extensively drug-resistant (XDR) TB, whose isolates are resistant to isoniazid and rifampicin and at least three of glycosides ([amikacin, kanamycin] or capreomycin, or both), polypeptides, fluroquinolones, thioamides, cycloserine and para-amino salicylic acid (CDC 2006).About 85% of the XDR isolates were from the KZN family of tuberculosis strains, which was described in 1996 (Davies *et al*., 1999). At that time, the KZN strains were either fully susceptible or had resistance to only first-line tuberculosis drugs. Resistance to second line drugs was not seen for the past 2-3 years (Sturum, 2006).

MOLECULAR MECHANISMS OF *M. TUBERCULOSIS* INFECTION AND HOST IMMUNITY

About 22% of the *M .tuberculosis* genes that were up regulated in activated versus resting macrophages were also up regulated in iron-depleted conditions. The gene *pyrG* (Rv1699), encoding CTP synthase is required for various metabolic pathways including cell membrane synthesis and the synthesis of phospholipids. The up-regulation of genes involved in mycothil, peptiglycan, phospholipids and other cell wall associated proteins suggests that these genes play an important role in *M. tuberculosis* protection from the host environment. In addition, a panel of genes involved in fatty acid oxidation including *Rv0215c*, *Rv0673*, *Rv0852*, *Rv2679* and *Rv3505* were induced during infection of resting and activated macrophages. Genes coding for regulatory proteins play vital roles in metabolic processes, including transcription, cell development and interactions with host cells. Regulatory genes that are up regulated during infection of both resting

and activated macrophages include *Rv0014c*, *Rv1534*, *Rv2640c*, *Rv2642*, *Rv3246*, *Rv3681*, *Rv0758*, *Rv0485* and *Rv0903c*. The greatest percentages of genes up-regulated in each condition investigated include both conserved hypothetical and hypothetical proteins of unknown functions (Rachman *et al*., 2006). A set of *M. tuberculosis* genes, which are required for its survival in macrophages, has been identified recently using a unique high throughout method combining Tra-SH and microarray methods (Rengarajan *et al*., 2005).

The cellular and molecular mechanisms of host defense against mycobacterial infection is understood a lot more today than a decade ago. Without understanding the nature of immune responses in human, the effort in hunting potentially better TB vaccines has to be broad-based. Macrophages are commonly regarded as the phagocytic cells that initially ingest *M. tuberculosis* and provide an important cellular niche during infection. The alveolar macrophages are the primary cells within the airway space including alveoli that phagocytosed mycobacteria. The abundant dendritic cells (DC) within the airway epithelium and the bronchial associated lymphoid tissue could also phagocytose mycobacteria as bacteria trans-cross the airway epithelium (Henderson *et al*., 1997; Schaible *et al*., 2000). Since monocytes and macrophages could differentiate into DC under correct conditions *in vitro* (Lane and Brocker 1999), it is possible that alveolar macrophages, after taking up mycobacteria, can differentiate into DC and then migrate into the lymph node. DCs are considered to be professional antigen presenting cells (APCs), due to their ability to endocytose antigens and express abundant quantities of MHC class II molecules and cytokines (Guermonprez *et al*., 2002). During the early stage, *M. tuberculosis* infected macrophages secrete pro inflammatory cytokines, interleukin-1, interleukin-6, interleukin-12 and tumor necrosis factor-α (TNF-α), as well as chemokines, which help to recruit monocytes,T-cells, B-cells and neutrophils to the site of infection. These recruited cells form granulomas, which are composed of centrally located macrophages and surrounded by T and B-lymphocytes.

Formation of such organized structures helps contain and prevent dissemination of infection and also allows for the close T-cell macrophage contact necessary for the induction of effective anti-mycobacterial mechanisms. At the cellular level, both CD4+ and CD8+ T cells are essential for the control of *M. tuberculosis* infection in humans. (Randhawa, 1990). CD4+ T cells, also called T-helper (Th) cells, provide T cell help to other immune cells, and thus amplify the immune response.CD4+ T cells are approximately 2-fold more abundant than CD8+T-cells at sites of *M. tuberculosis* infection, and depletion or deletion of CD4+ T cells has an earlier and more striking effect than elimination of CD8+ T cells on the control of *M. tuberculosis* in mice (Mogues *et al*., 2001).Th1 polarized CD4+ effector cells are much more important than Th2 cells for control of tuberculosis. The major aspect of the importance of Th1 cells in tuberculosis host defense is their ability to secrete interferon-γ (IFN-γ). CD4+ T cells probably constitute the major source of this protein. Production of both reactive oxygen intermediates and reactive nitrogen intermediates by macrophages are stimulated by interferon-γ. This also effects the expression of Major Histo compatibilty Complex (MHC) class II molecules, thus leading to increased antigen presentation and further amplification of the immune response.

Initial studies focused on the role of CD4+T cells in tuberculosis host defense, but in recent years a great deal of attention has been devoted to CD8+ T cells with regard to protective immunity. CD8+ T cells can also bind the CD1 molecule, a more recently described mode of antigen presentation, which is present on the surface of professional antigen presenting cells. CD8+ cells can be both cytotoxic, causing lysis of infected target cells such as monocytes and macrophages and microbicidal causing death of intracellular pathogens directly. A nonamer epitope from the *M. tuberculosis* protein ESAT-6 could be recognized by CD8+ T cells for long periods of time after infection. These data

suggest that long lived specific CD8+ T cell responses are associated with the control of *M. tuberculosis* infection in humans (Pathan *et al*., 2001). The relative importance of CD4+ and CD8+ T cells in the immune response must be clarified, although gathering evidence suggests that the former play a greater role in the immune response against active disease and that the latter are more involved in controlling latent infection.

GENETIC PREDISPOSITION OF HOST

Several observational studies indicate that certain populations appear to exhibit unusual susceptibility to tuberculosis and are likely that to certain degree this susceptibility has a genetic basis. This idea has arisen from studies among monozygotic and dizygotic twins (Comstock, 1978) and in an assessment of tuberculosis risk according to ancestral history (Stead, 1992). Polymorphisms in several candidate genes have been linked to relatively increased risk for tuberculosis disease (Hill, 1998). These genes include several human leukocyte antigen loci (HLA alleles), Vitamin D receptors and the gene for the natural resistance-associated macrophage protein (NRAMP) and interleukin-1 (Bellamy *et al*., 1999; Greenwood *et al*., 2000). *NRAMP1* polymorphisms could influence tuberculosis susceptibility by regulation of interleukin 10 (Awomoyi *et al*., 2002). A human homologue for NRAMP (*NRAMP1*, alias *SLC11A1*) was implicated in developing active tuberculosis among patients with latent infection in West Africa (Bellamy *et al*., 1998) and also for the susceptibility to both primary and reactivated TB (Malik *et al*., 2005). Associations between genetic polymorphisms and tuberculosis susceptibility differ according to ethnic origin (Delgado *et al*., 2002), but to which extent genetic polymorphisms contribute to the global tuberculosis burden is unclear because of the great difficulty of separating lifelong environmental influences from genetic predisposition.

DIAGNOSIS OF TUBERCULOSIS

The rapid and accurate diagnosis of tuberculosis (TB) is crucial for effective treatment to reduce morbidity and mortality associated with it. For decades, the gold standard in diagnosis has relied on the microscopic examination of sputum smears and culture of bacteria combined with clinical examination (Pottumarthy *et al*., 1999). The purified protein derivative (PPD) skin test is an important tool for diagnosis of TB which contains crude antigens, many of which are shared among non-tuberculosis mycobacteria (Andersen *et al*., 2000; Lee and Holzman, 2002). As a result, the tuberculin skin test (TST) has lower specificity in populations with high BCG coverage and non-tuberculous mycobacteria (NTM) exposure. The sensitivity may be low in individuals with depressed immunity such as AIDS and other immunosuppressive conditions (American Thoracic Society, 2000; Jasmer *et al*., 2002). This has prompted the need to develop rapid, inexpensive but clinically sensitive and specific tests that can improve upon current diagnostic tests (Foulds and Brien, 1998). PCR methods are available for field use (Miller *et al*., 1994; Noordhoek *et al*., 1994). Barry *et al*. (2003) concluded by their study that the PPD activated CD4+ T cells type 1 cytokine response, measured by IFN-γ or TNF-α synthesis appears to be a useful measure for diagnosis of active TB in HIV infected individuals. The Quanti FERON-TB assay (Cellestis Limited, Carnegie, Victoria, Australia) and the T-spot TB assay (Oxford Immunotec, Oxford, United Kingdom) are two commercial IFN-γ assays.

The diagnosis of TB has been facilitated by serological enzyme-linked immune sorbent assay (ELISA) and immno chromatographic analysis (ICA) with thermostable macromolecular antigens (TMAs) which are immunologically active complexes present in all mycobacteria (Zou *et al*., 1994). Through gene cloning, expression and peptide synthesis, defined antigens can be produced *in vitro* for use in serodiagnosis. Many attempts have been made to identify and isolate

specific antigens from *M. tuberculosis* for use as diagnostic reagents (Turcotte, 1975; Nagai *et al*., 1991). The identification of regions in the *M. tuberculosis* genomeare regions that are absent in BCG and most non-tuberculous mycobacteria (Gordon *et al*., 1999; Behr *et al*., 1999) and provides an opportunity to develop new specific diagnostic tools by rational design. The assays based on region of deletions (RD1) antigen are more specific than TST and PPD based assays. Anin-house enzyme linked immunospot (ELISPOT) IFN-γ assay based on a restricted pool of highly selected synthetic peptides derived from ESAT-6 and CFP-10 proteins has been recently validated. The test was highly specific (87%) and sensitive (93%) and made it possible to distinguish individuals infected with *M.tuberculosis* from those vaccinated with BCG (Scarpellini *et al*., 2004; Codecasa *et al*., 2006).

A rapid molecular test known as Xpert MTB/RIF (automated, cartridge- based nucleic amplification assay) can diagnose TB and rifampicin resistance directly from sputum in an assay that provides results within 100 minutes, has been endorsed by WHO in December 2010 and at the end of June 2012, 1.1 million tests had been purchased by 67 low- and middle-income countries; South Africa (37% of purchased tests) is the leading adopter. A 41% price reduction (from US$ 16.86 to US$ 9.98) in August 2012 should accelerate uptake. Results from field demonstration studies found that a single Xpert MTB/RIF test can detect TB in 99% of patients with smear-positive pulmonary TB and >80% of patients with smear-negative pulmonary TB. The demonstration studies also showed that while HIV co infection substantially decreases the sensitivity of microscopy, it does not significantly affect the performance of Xpert MTB/RIF. Furthermore, Xpert MTB/ RIF can detect rifampicin resistance with 95.1% sensitivity and exclude resistance with 98.4% specificity. The test has the capacity to revolutionize the diagnosis of TB and drug-resistant TB, since it can greatly increase case finding and overcomes several barriers to establishing diagnostic capacity at country level, including human resource and biosafety constraints (WHO Report, 2012).

TREATMENT

Even if one effective vaccine is developed, it might not prevent progression to active disease among the more than two billion people already infected with *M. tuberculosis*. Therefore, more effective treatment systems would be required for decades. The goals of treatment are to ensure cure without relapse, to prevent death, to stop transmission, and to prevent the emergence of drug resistance. Almost all recommended treatment regimens have two phases (CDC, 2003; JTC-BTS, 1998). At least two bactericidal drugs, isoniazid and rifampicin, are necessary in the initial phase, which is designed to kill actively growing and semi dormant bacilli. The addition of ethambutol benefits the regimen when initial drug resistance may be present or the burden of organisms is high. Directly Observed Treatment Short course (DOTS) is highly effective at promoting successful treatment and is recommended by WHO and IUATLD (WHO, 2003; Enarson *et al*., 2000). In the USA, pyrazinamide is not recommended for use during pregnancy except when alternative drugs are not available or are less effective (CDC, 2003). Advised treatment regimens are similar for HIV infected and HIV-negative tuberculosis patients. However, thioacetazone should never be used, because it is associated with an increased risk of severe and in some cases fatal skin reactions in HIV infected individuals (Nunn *et al*., 1991; Okwera *et al*., 1994). Treatment success in XDR tuberculosis is very difficult as only few drugs remain active. Thus, drug resistance tuberculosis and its nosocomical transmission threaten the achievements of DOTS and anti-retroviral scale-up programmes, which are now widely being implemented in resource-limited settings worldwide.

THE CURRENT VACCINE, BACILLUS CALMETTE-GUERIN (BCG) AND THE REASON FOR ITS FAILURE

The Bacillus Calmette-Guerin (BCG) consists of clonal bacterial strains, which were developed by Albert Calmette and Camille Guerin. It is the only TB vaccine currently in use. These vaccines are mutated form of the causative agent of bovine TB, *Mycobacterium bovis*, which became attenuated between 1908 and 1921 while being cultivated on beef-bile-potato medium in Calmette's laboratory at the Pasteur Institute (Oettinger*et al*., 1999). From the introduction of virulent *M. bovis* into the laboratory in 1908 to the lyophilization of BCG by Pasteur in 1961, BCG has endured 53 years of 1173 passages of laboratory selective pressures with an estimate of some 15,000 generations elapsed between the original manipulation of *M. bovis* and immortalization.

BCG has been used for 80 years and is now the most widely administered vaccine with a global coverage of 81% of world population (WHO Global summary, 2000). BCG vaccines (many genetically different sub strains) are given to over 120 million persons each year. This vaccine shows a varied success rate (0% to 80%) in preventing tuberculosis (Colditz *et al*., 1994; Harboe *et al*., 1996; Fine, 1995; Olsen *et al*., 2000; Coler *et al*., 2001). The South Indian BCG trial report has shown that BCG did not offer any protection against the adult form of bacillary pulmonary TB (TRC-ICMR, 1999). Randomized and case control trials have been shown consistent high protective efficacy (mostly above 70%) of BCG, against only the serious forms of disease in children such as meningitis (Fine, 2000). Thus, in high prevalence areas vaccination is recommended for children at birth except for children with symptomatic HIV infection (WHO, 2002). The choice of the BCG strain to be used for vaccination is a very important issue. It is currently difficult to determine which strain should be used, and further detailed analysis of the genomics and immunogenicity of BCG sub strains may provide an answer to this important question.

The activity of the neonatal vaccination wanes after 10-12 years and the incidence of contagious pulmonary TB increases in adolescence. The current global scourage of TB speaks for the ineffectiveness of BCG vaccine and that could be multifactorial. Several reasons for its variable efficacy have been proposed, ranging from the influence of prior infection with environmental mycobacteria to the absence of antigens that are protective against *M. tuberculosis* (Harboe *et al*., 1996; Brandt *et al*., 2002). Gene deletion was an important part of the attenuation process that led to the BCG vaccine. The protective immunity by BCG requires bacterial replication in the host, which is blocked by a pre-existing immune response cross-reacting to BCG, thereby limiting the development of a protective immune response. The failure of BCG in sensitized individuals also impose the important limitation that BCG cannot be used successfully as a booster vaccine to boost the waning effect of the BCG vaccination given after the birth (Dietrich *et al*., 2006). Geographical latitude was the biggest single factor explaining the variable efficacy (Fine, 1995).

The present hypotheses for the failure of BCG in developing countries include the following: the rapid destruction of BCG by T helper 1(TH1)-cell responses that are elicited by cross-reactive mycobacteria; the presence of TH2-cell responses, which are driven by co-existing helminth infections; malnutrition; the variations evolved in the strains of BCG that are used and strains of *Mycobacterium tuberculosis* that are encountered. BCG fails to adequately stimulate CD8+ T Cells, probably because it has lost essential cytolysins. Genetic differences in human ethnic groups leading to different mechanisms of action of BCG in different races were postulated as yet another reason why BCG failed to show any protection in some populations (TRC-ICMR, 1999). Comparative genomics identified >100 coding sequences being absent

from BCG but present in *M. tuberculosis* (Behr *et al*., 1999; Lewis *et al*., 2003). This leads to failure to mimic S*M. tuberculosis* when exposed to a human host. Some of these coding sequences encode potential antigens that could improve immunogenicity if reintroduced into BCG (Pym *et al*., 2002).

REGION OF GENE DELETIONS – RD1 to RD16 AS GENETIC HOTSPOTS FOR THE SERODIAGNOSIS OF *M. TUBERCULOSIS* INFECTION

The genomes of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* and the attenuated *M. bovis* BCG have been compared using variety of methods like substractive genomic hybridization (Mahairas *et al*.,1996), DNA microarrays (Behr *et al*., 1999) and BAC arrays (Brosch *et al*., 2000). Several regions of deletions (RDs) were identified among these organisms. These regions were designated as RD1-RD16 and encompassing 129 open reading frames varying in length from 1903 to 12,733 base pairs (Brosch *et al*., 2000). The loss of the regions RD2, RD8, RD14 and RD16 in BCG could have been responsible for a decrease of protective immunity induced by these BCG sub strains (Behr and Small, 1999). Region of deletion 2 (10.7 kb DNA) was conserved in all virulent laboratory and clinical tubercle bacilli, but deleted from those BCG sub strains that were derived from the original BCG Pasteur strain after 1925. However, the antigens of vaccination studies performed with different BCG sub strains, both in mouse and man, do not show the influence of RD2 encoded antigen(s) on protection against TB (Brewer and Colditz, 1995; Colditz *et al*., 1995). RD3 (9.3 kb DNA), is present in the virulent laboratory strains of *M. bovis* and *M. tuberculosis*, but deleted in all sub strains of BCG. Moreover, RD3 is also deleted from the genome of 84% clinical isolates of *M. tuberculosis* and therefore the antigens expressed from this region will not be important for developing new vaccines or diagnostic reagents against TB (Mahairas *et al*., 1996).

The RD1 region is the best characterized of the deleted regions. It has been long speculated and recently been confirmed that the 9455 bp region of deletion 1 (RD1) of *M. tuberculosis* contributes to bacterial virulence (Pym *et al*., 2002; Lewis *et al*., 2003). This region is commonly thought to be the primary deletion that occurred during the serial passages of *M. bovis* by Calmette and Guerin between 1908 and 1921.This gene segment is found to be absent from all BCG sub strains and most environmental mycobacteria but is present in all strains within the *M. tuberculosis* complex. Reintroduction of this region into BCG led to a protein expression profile almost identical to that of virulent *Mycobacterium bovis* and *M. tuberculosis* as assessed by proteomics (Mahairas *et al*., 1996). Consequently, the genes contained in RD1 have been the object of a number of studies focusing on diagnosis of *M. tuberculosis* infection, the search for efficient vaccine candidates and virulence (Arend *et al*., 2000; Wards *et al*., 2000).

RD1 encompasses the genes *Rv3871* to *Rv3879c* (annotation according to Cole *et al*., 1998), which includes the genes for the 6-kDa early secretory antigenic target (ESAT) and L45 homologous culture filtrate protein-10 (CFP-10) (Andersen *et al*., 1995; Berthet *et al*., 1998). The RD1 deletion in BCG disrupts nine coding sequences and affects all four transcriptional units. Transcriptional analysis of BCG using DNA micro arrays indicated that the *Rv3867-Rv3871* transcriptional units were still active. *Mycobacterium tuberculosis* clinical isolate MT56 does not have *Rv3878* or *Rv3879* but still secretes ESAT-6 and CFP-10, whereas BCG and *M. bovis* have frame shifts in *Rv3881* (Pym *et al*., 2003). The identification of RD1 (Mahairas *et al*., 1996) and the subsequent demonstration of its role in the virulence of *M. tuberculosis* (Lewis *et al*., 2003) have fueled the speculation that this region could be exploited for the development of a vaccine for TB (Pym *et al*.,2003; Hsu *et al*., 2003). Therefore RD1 gene products can be targeted for the development of

new diagnostic tests to differentiate *M. tuberculosis* infection from BCG vaccination and exposure to environmental mycobacteria (Lalvani *et al*., 2001).

RD1 ANTIGENS

Based on the genetic variability within strains of BCG, it is interesting that RD1 is the only region absent in all strains of BCG (Gordon *et al*., 1999). This region encodes several proteins that are expressed early during infection, including the ESAT-6 and CFP-10 (Berthet *et al*., 1998). It was suggested that IFN-γ assays that use *M. tuberculosis* specific region of deletion antigens may have advantages over the TST, in terms of higher specificity, less cross reactivity due to BCG vaccination and non-tuberculous mycobacterial infection (Pai *et al*., 2004). Assays that use cocktails of RD1 antigens seem to overcome this problem, and such assays have the highest accuracy. RD1 based IFN-γ assays can potentially identify those with latent tuberculosis who are not at high risk for developing active disease. Among the missing genes are the adjacent coding sequences *esxB* (CFP10) and *esxA* (ESAT6), low molecular proteins that induce potent Th1 responses (Berthet *et al*., 1998; Colangelli *et al*., 2000; Coler *et al*., 2001).

Included in the RD1 locus, the *esx* genes are part of a conserved segment that arouse by duplication, encoding members of the five additional protein families (Cole *et al*., 1998; Tekaia *et al*., 1999). The precise function of the most of the coding sequences in these regions are unknown, but their linkage with *esx* genes suggests they could encode a protein complex required for the export of ESAT-6 proteins (Van Pittius *et al*., 2001). *In vivo*, ESAT-6 and CFP-10 is associated as a heterodimer in a tight 1:1 complex (Renshaw *et al*., 2002). Although no direct function has been attributed to these proteins, their expression correlates with an increased cytolytic ability of *M. tuberculosis* (Hsu *et al*., 2003). These proteins are recognized by human, murine, and bovine T cells (Brodin *et al*., 2004), which respond by proliferating and secreting gamma interferon (IFN-γ) (Andersen *et al*., 1995; Skjot *et al*., 2000). The *lhp* and *east6* genes as well as other genes are part of a cluster called the "ESAT6 superfamily" which is found in multiple copies in several mycobacterial genomes (Tekaia *et al*., 1999) and may contain a novel secretion apparatus (Pallen, 2002).

Significant immunological activity of the proteins encoded by the RD1 region of *M. tuberculosis* was recently supported by the identification of the third RD1 protein TB27.4 encoded by *rv3878* Open Reading Frame (Aagaard *et al*., 2003). According to the Sanger database, *rv3878* from the RD1 region of *M. tuberculosis* codes for a hypothetical alanine rich TB27.4 protein with a molecular mass of 27000 Daltons. Similarly, Brusasca *et al*. (2001) analyzed the products of six Open reading frames in the RD1 region *Rv3871, Rv3872, Rv3873,* MTSA-10, ESAT-6 and *Rv3878.* The serological reactivity of these antigens in human TB was evaluated. The absence of these antigens with sera from mycobacteria other than tubercular (MOTT) patients is consistent with the observed lack of homologues for these proteins in *M. avium*, which is the commonest cause of non-TB mycobacterioses in humans (Grange, 1996).

Combination of Rv1986, Rv3872 and Rv3878 indicated that 82% of the infected animals would have been correctly identified by their responses to these three antigens and they considered suitable for differential diagnosis. Interestingly, Rv3878 together with Rv3873 and Rv3879c detected 21 (95%) of the 22 *M. bovis* infected animals, a figure identical to that for the ESAT-6 / CFP-10 cocktail (Cockle *et al.,* 2002).Moreover, based on the observation, the major T-cell targets from short term culture filtrate in *M. bovis* infected cattle are also the immunodominant T-cell antigens which may prove to be similarly potent targets of cell mediated immune responses (CMI) in TB patients (Pollack and Andersen 1997). Since, *M. bovis* has greater than 99.9% DNA sequence identify with *M. tuberculosis,* antigens thus identified in

cattle will have amino acid sequences almost identical to those of their *M. tuberculosis* counterparts and can therefore be directly tested in humans(Hewinson*et al*., 2003).

Liu *et al.* (2004) selected four gene products on the basis of the following criteria. First, they were all strong targets of CMI responses in a high proportion of *M. bovis* infected cattle (Cockle *et al*., 2002). Second, they are present in *M. tuberculosis* as well as *M. bovis*. Third, they are absent for most (*Rv1989c* from RD2) or all (*Rv3873*, *Rv3878* and *Rv3879c* from RD1) strains of BCG (Behr *et al*., 1999). To further investigate the immunological response to four other predicted proteins from the RD1 region which was studied in cattle, Agger *et al*. (2003) studied the human response to one of these predicted proteins encoded by *rv3878* (TB27.4). The immunological reactivity to rTB27.4 was evaluated by stimulating T-cell lines as well as PBMC obtained from TB patients.rTB27.4 induced positive responses in 9 out of 12 T-cell lines with a mean IFN-γ levels of 285pg/ml (with a cut off level of 100 pg/ml). No reactivity to rTB27.4 was seen in the group of BCG vaccinated or non-vaccinated donors confirming the specificity of the antigen.

The RD1 antigens tested so far, including TB27.4, contained numerous epitopes distributed throughout the molecule (Ravn *et al.,* 1999: Arend *et al.,* 2000). This enables the generation of a multipeptide cocktail for the broad spectrum of HLA polymorphisms seen in genetically different populations. In protein RV3878, two specific regions were selected, peptides 3 to 9 (Rv3878A) and peptides 11 to 15 (Rv3878B). By combining Rv2654, Rv3873A and Rv3878B peptides a sensitivity of 57% (95%CI, 42to 72%) was obtained. This sensitivity level is not significantly different from the sensitivity obtained with ESAT- 6 or CFP-10 (75% and 66%, respectively) (Brock *et al.,* 2004). In this context, it can be mentioned that Rv3872 and Rv3878 could discriminate extra-pulmonary TB patients from healthy BCG vaccinated individuals. For Rv3878, the sensitivities of detection were 88% and 84%, in pulmonary and extra pulmonary TB patients, respectively. For Rv3872 and Rv3878 the specificities were 95% and 91% respectively (Mukherjee *et al.,* 2006). Compared with CFP-10 and ESAT- 6 the differences in mean absorbance values among TB patients and healthy BCG-vaccinate individuals were much higher for Rv3872 and Rv3878. Furthermore, Mukherjee *et al*., (2007) showed that the Rv3878 protein elicited strong humoral immune response in TB patients in a selected Indian TB population, supporting the notion that the antibody response to a specific antigen often depends on the geographical location and ethnic background of the population being studied.

RD1 ANTIGEN COCKTAILS – AS SERODIAGNOSTIC MARKERS

Baassi *et al*., (2009) assessed the potential use of the *in silico* selection approach of *M. tuberculosis*-specific synthetic peptides in the serodiagnosis of active TB. Using both multivariate logistic regression analysis and the random forest method, they could identify a pool of nine peptides belonging to the proteins Rv0747, Rv1114, Rv1434, Rv1979c, Rv3874, Rv3736, Rv3878 and Rv3883 as the best combination of peptides discriminating between TB patients and healthy controls. The study also proposed a peptide belonging to the *M. tuberculosis* protein Rv3878 as the peptide that best discriminates between TB patients and healthy controls in a population with low exposure to TB.

Dosanjh *et al*., (2011) investigated T-cell responses to Rv3873, Rv3878 and Rv3879c after tuberculosis exposure and correlated responses with progression to active tuberculosis disease and established markers of recent infection, i.e. TST conversion and ESAT-6/ CFP-10-ELISpot conversion, in a well characterized cohort of children with a very low prevalence of HIV infection and recent household tuberculosis exposure. With no gold standard test for Latent Tuberculosis Infection (LTBI), progression to active disease is the only way to confirm *M. tuberculosis* infection and is

essential for defining the clinical utility of new biomarkers of LTBI. Baseline T-cell responses specific for Rv3873 and Rv3878 significantly predicted progression to active tuberculosis, indicating that the T-cell responses to these novel antigens are not only specific, but also prognostic of *M. tuberculosis* infection. These are the first antigens, other than those used in the commercial IGRAs, to be identified as having prognostic value.

The genes encoding CFP-10, ESAT-6 and PPE68 are situated adjacent to each other in the *M. tuberculosis* genome, suggested that they may constitute a gene cluster that encodes a new transporter system in *M. tuberculosis*. The frequent occurrence of T-cell determinants in this region suggests that this functional unit may be particularly active in interacting with the host immune system and may play an important role in host-pathogen interactions (Tekaia *et al*., 1999). Findings suggested that when the fusion protein CFP-10–ESAT-6–PPE68 as a targeted pathogenic agent challenges the body, immunological memory can be induced quickly. ELISA results clearly demonstrated that the purified fusion protein CFP-10–ESAT-6–PPE68 has both sensitivity and the specificity better than those of CFP-10–ESAT-6 and PPD in the active-TB patients and controls (Xu *et al*., 2012).

The MTB-48 (rv3881c) a 1,383 bp open reading frame (ORF) encodes a predicted 47.6 kDa protein. The genomic location of this sequence is 826 bp upstream of region containing the esat-6 gene that is deleted in the *M. bovis* BCG isolate. This sequence is present as a single copy and is highly conserved in the *M. tuberculosis* and *M. bovis* isolates tested, and between geographically isolated strains form the United States and India but not in other mycobacterial species tested, including *Mycobacterium leprae* and *Mycobacterium avium*. The native protein is cytoplasmic and not membrane bound, the truncated form of the native protein is found both in the cytoplasm and in the culture filtrate. Preliminary epitope mapping and protein analysis shows that the amino terminus and central region of the protein are hydrophilic and are predicted to be highly antigenic.

Four recombinant proteins representing the amino (rTbH4) and carboxy (rXP-1) termini, the full length sequence (rMTB- 48) and the mature polypeptide for the *M. tuberculosis* 38 kDa antigen (r38kDa) were evaluated by ELISA. rTb H4 was reactive in 19 of 19 serum samples, while rXP-1 was reactive with 11 of 19 serum samples. These data indicate that approximately 42% of MTB-48 positive patient serum samples are reactive only with an amino-terminal epitope(s). While 58% of the serum samples are reactive only with central epitope(s) (Lodes *et al*., 2001). Overall sensitivities for patients with only *M. tuberculosis* infection were 29.8% and 43.4% for rMTB-48 and r38kDa respectively. The sensitivity when the antigens were used in combination, however, was 56.3% indicating a gain of 12.9% over rMTB48 alone. Although rMTB adds to the overall sensitivity and specificity will depend on the use of several antigens, including rMTB48. In general, *M. tuberculosis* antigens with serodiagnostic potential can be divided into six categories based on the type of TB they preferentially detect. A combination of recombinant proteins and a fusion polyprotein which may detect different forms of TB (caused by different strains and in different host populations): the 38-kDa antigen, MTB8, MTB11, MTB48, MTB81, DPEP, TB9.7 and TB16.3 (Abebe *et al*., 2007).

Kulshrestha *et al*., (2005) described optimized protocols for the expression and purification of soluble and monomeric ESAT-6, CFP-10, MTC-28 and α-crystallin (14-kDa antigen) of *M. tuberculosis* and were used in ELISA for detecting antibodies in sera of patients with tuberculosis. These four antigens did not show high sensitivity with sera from different categories of TB patients. However, each of these four antigens detected several samples which were not detected by a combination of 38-kDa and MTB81 antigens. It is important to note that highly pure antigens obtained showed very

low reactivity with normal healthy donors. These results indicated that use of these antigens along with 38-kDa and MTB-81 antigens should increase the sensitivity of detection in various categories of TB patients without jeopardizing the specificity. Zhang *et al.*, (2012) successfully recombined five RD5-encoded proteins and evaluated for their diagnostic potential in detecting serum antibodies comparison with ESAT-6 antigen with sera from HIV-negative pulmonary TB patients and healthy control subjects. These results identified two immunodominant antigens Rv3117 and Rv3120, which show promise for use in the serodiagnosis of *M. tuberculosis*. They both revealed a statistically significant antigenic distinction between healthy controls and TB patients. Rv3881c and three other antigens predicted to be lipoproteins (Rv0934 (pstS1), Rv0932c (pstS2) and Rv3006 (LppZ)) and the most immunogenic taken into consideration the low amount of proteins present in the culture filtrate. Yet a significant relative amount of specific antibodies present in the TB patients' serum. This illustrates the significance of lipoproteins as potential diagnostic markers, and they should certainly be investigated further (Malen, 2008).

TARGETING THE PE AND PPE GENE FAMILIES OF *M. TUBERCULOSIS*

One of the major findings of the *M. tuberculosis* genome project was the identification of large gene families, which are the novel PE and PPE families, comprising 100 and 67 members, respectively. Genome sequencing of the *M. tuberculosis* has revealed that approximately 10% of the genome encodes two families of glycine rich proteins termed PE and PPE on the basis of their characteristic Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs near the N-terminal domain (Cole *et al*., 1998; Cole and Barrel, 1998).The PE and PPE families can be divided into subfamilies on the basis of their repetitive sequence and complexity. The PE family has been classified into several subfamilies. The largest of these is the polymorphic repetitive sequence class (PGRS), characterized by high glycine content, and also referred to as fibronectin binding proteins (Abu-Zeid *et al*., 1991; Espitia *et al*., 1999). It has been suggested that some PE proteins may play a role in immune evasion and antigenic variation (Banu *et al*., 2002; Brennan and Delogu, 2002) and some members have been found to be associated with the cell wall (Delogu *et al*., 2004). The PPE proteins are likely to be antigenically polymorphic and it has been speculated that they may have an important immunological significance, as a source of antigenic variation or by inhibiting antigen processing (Cole *et al*., 1999; Choudhary *et al*., 2003). Whole genomes comparison and functional genomics have shed new light on the possible roles of the PE and PPE proteins. Furthermore, because the PE and PPE genes are prevalent in *M. tuberculosis* and absent in humans, they may serve as potential targets for the development of anti-tuberculosis intervention strategies.

CONCLUSIONS

Both genetic and environmental factors influence the antibody response in *M. tuberculosis* infection. For example, the varied antibody response to *M. tuberculosis* is managed by human leukocyte antigen types. Therefore, the fact that some pool of peptides identified in one population has strong diagnostic value than another population with a different genetic background suggests that these peptides may form the basis of a test that can be used independently of location. Attempts to develop accurate serodiagnostic tests for TB have been unsuccessful. Whereas antibody responses to mycobacterial antigens in adult TB have been studied extensively and reviewed, the same cannot be said for serologic data in pediatric populations. It was also concluded that the limitations in serodiagnostic studies of childhood TB are manifold, thereby restricting the interpretation of currently available data. Concerns about the methodology used in published studies suggest that conclusions about the eventual value of serodiagnosis cannot be made at this time. However, the available data

suggest a potential adjunctive value for serology in the diagnosis of childhood TB. Despite the difficulties noted in this field, there is optimism that the application of novel antigens and the integration of those factors which contribute to the serological responses in childhood TB can lead to useful future diagnostics. In summary, given all the difficulties in diagnosing pediatric TB, serology remains a very attractive diagnostic approach. Future studies with immunodominant mycobacterial antigens in age defined subgroups are needed to establish the usefulness of serology in diagnosis and move the field forward.

REFERENCES

1. Aagaard, C., M. Govaerts, L. M. Okkels, P. Andersen, and J.M. Pollock (2003). Genomic approach to the identification *Mycobacterium bovis* diagnostic antigens in cattle. *J. Ciln. Microbiol.* 41: 3719-3728.
2. Abebe, F., C. Holm-Hansen, H.G. Wiker and G. Bjune (2007). Progress in Serodiagnosis of *Mycobacterium tuberculosis* infection. *Scand. J. Immunol.* 66: 176-191.
3. Abu-Zeid, C., T. Garbe, R.Lathigra, H.G. Wiker, M. Harboe*et al*. (1991). Genetic and immunological analysis of *Mycobacterium tuberculosis*fibronectin -binding proteins. Infect. Immun, 59(8), 2712-2718.
4. Agger, E.M., I. Brock, L.M. Okkels, *et al*. (2003). Human T-cell responses to the RD1 encoded protein TB27.4 (Rv3878) from *Mycobacteriumtuberculosis*. *Immmunol.* 110: 507-512.
5. American Thoracic Society (2000). Diagnostic standards and classification of tuberculosis in adults and children. Am. J. Respir.Crit. Care. Med, 161, 1376-1395.
6. Andersen P., M.E. Munke, J.M. Pollock and T.M. Doherty (2000). Specific immune based diagnosis of tuberculosis. *Review.* The Lancet, 356, 1099-1104.
7. Andersen, P., A.B. Andersen, A.L Sørensen and S. Nagai (1995). Recall of long-lived immunity to *Mycobacterium tuberculosis* infection in mice. J. Immunol, 154, 3359-3372.
8. Arend, S.M., A. Geluk, K. E. VanMeijgaarden, J. T. van Dissel, M. Theisen, P. Andersen and T. H. M. Ottenh off (2000). Antigenic equivalence of human T-cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10, and those to mixtures of synthetic peptides. Infect. Immun, 68, 3314-3321.
9. Awomyi, A., A. Marchant, J. M. M. Howson, K.P. Mc Adam, J.M. Blackwell, and M.J. Newport (2002). Interleukin-10, polymorphism LC11A1 (formerly NRAMP1), and susceptibility to tuberculosis. J Infect. Dis, 186, 1808-1814.
10. Banu,S., N. Honore, B. Saint-Joanis, D. Phillpot, D. Prevost and S.T. Cole.(2002). Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? Mol. Microbiol, 44, 9-19.
11. Barry, S.M., M.C. Lipman, B. Bannister, M.A. Johnson and G. Janossy (2003). Purified protein derivative-activated type-1 cytokine - producing CD4+ T lymphocytes in the lung: a characteristic feature of active pulmonary and nonpulmonary tuberculosis. J. Infect. Dis, 187, 243-250.

12. Behr, M.A. and P.M. Small (1999). A historical and molecular phylogeny of BCG strains. Vaccine, 17, 915-922.

13. Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G.K. Schoolnik *et al*.(1999). Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science, 284, 1520-1523.

14. Bellamy, R., C. Rewende, T. Corrah, et al. (1999). Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. J. Infect. Dis, 179, 721-724.

15. Bellamy, R., C.Ruwende, T .Corrah, K.P. Mc Adam and H.C. Whittle (1998). Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. N. Engl. J. Med, 338, 640-644.

16. Berthet, F.X., P.B. Rasmussen, P. Andersen, I. Rosenkrands, and B.A. Gicquel (1998). *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiol, 144, 3195-3203.

17. Betts, J.C., P.T. Luckey, L.C. Robb, R.A. Mc Adam& Duncan, K.(2002).Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol. Microbiol., 43, 717-731.

18. Brandt. L., J.F.Cunha, A.W.Olsen, B.hilima, P.Hirsch, R.Appelberg and Andersen(2002). Failure of the *Mycobacterium bovis* BCG Vaccine: Some Species of Environmental Mycobacteria Block Multiplication of BCG and Induction of Protective Immunity to Tuberculosis. Infect. Immun, 70(2), 672-678.

19. Brennan, M.J. and G. Delogu (2002). The PE multigene family: a 'molecular mantra' for mycobacteria. Trend. Microbiol, 10, 246-249.

20. Brewer, T.F. and G.A. Colditz (1995). Relationship between BacilleCalmette-Guerin strains and the efficacy of BCG vaccine in the prevention of tuberculosis. Clin. Infect. Dis, 126-135.

21. Brock, I., K. Weldingh, E. M. S. Leyten, S.M. Arend, P. Ravn *et al*. (2004). Specific T-cell epitopes for immunoassay-based diagnosis of *Mycobacterium tuberculosis* infection. *J. Clin. Microbiol*. 42(6): 2379-2387.

22. Brodin, P., I. Rosenkrands, P. Andersen, S.T. Cole and R. Brosch. (2004). ESAT-6 proteins: protective antigens and virulencefactors?TrendsinMicrobiology, 12(11),500-508.

23. Brosch, R., S.V. Gordon, A. Pym, T. Garnier K. Eiglmeier and S. T. Cole (2000a). Comparative genomics of the mycobacteria. Int. J. Med. Microbiol. 290, 143-152.

24. Brosch, R., S.V.Gordon, C. Buchrieser, A. S. Pym, T. Garnier *et al*. (2000b). Comparative genomic uncovers large tandem chromosomal duplications in *Mycobacterium bovis* BCG Pasteur. Yeast. 17, 111-123.

25. Brusasca,P.N., R.Colangeli, X.Zhao, K.P. Lyashchenko, M. Vogelstein *et al*. (2001). Immunological characterization of antigens encoded by the RD1 region of *Mycobacterium tuberculosis* genome. *Scand. J. Immunol*. 54: 448-452.

26. CDC, CDC-USA (MMWR) (2006). Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs worldwide, 2000-2004. pp. 301- 305.

27. Choudhary,R.K., S. Mukhopadhyay, P. Chakhaiyar, N. Sharma, K.J.R.Murthy *et al*.(2003). PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B cell response. Infect. Immun,71, 6338-6343.

28. Cockle, P.J., S. V. Gordan, A. Lalvani, B. M. Buddle, R. G. Hewinson and H. M. Vordermeier (2002). Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infect. Immun.* 70(12): 6996-7003.

29. Codecasa, L., P. Mantegani, L. Galli, A. Lazzarin, P. Scarpellini*et al*. (2006). An In-house RD1-based enzyme linked immunospot-gamma interferon assay instead of latent *Mycobacterium tuberculosis* infection. J. Clin. Microbiol, 44(6), 1944-1950.

30. Colangeli, R., J.S. Spencer, P. Bifani, A. Williams, K. Lyashchenko, M.A. Keen, P.J. Hill, J. Belisle and M.L. Gennaro (2000). MTSA-10, the Product of the Rv3874 Gene of *Mycobacterium tuberculosis*, elicits tuberculosis -specific, delayed-type hypersensitivity in Guinea Pigs. Infect. Immun, 68(2), 990-993.

31. Colditz,G.A., T. F. Brewer, C. S. Berkey, M.E. Wilson, E. Burdick, H.V. Fineberg and F. Mosteller (1994). Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA, 271, 698-702.

32. Colditz, G. A., C. S. Berkey, F. Mosteller,T. F. Brewer, M.E. Wilson *et al*.(1995). The Efficacy of BacilleCalmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis. Meta-analysis of the published literature. Pediatrics, 96, 29-35.

33. Cole, S.T. and B.G. Barrell (1998). In genetics and tuberculosis (Eds: D.J.ChadwickandG. Cardew, Novartis foundation symp. 217, Wiley, Chichester, pp.160-172.

34. Cole, S.T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher*et al*. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature,393, 537-544.

35. Coler, R.N., A. Campos-Neto, P. Ovendale, F.H. Day, S.P. Fling, L. Zhu, N. Serbina, J.L. Flynn, S.G. Reed and M.R. Alderson (2001). Vaccination with the T cell antigen Mtb-8.4 protects against challenge with *Mycobacterium tuberculosis*, J. Immunol, 166, 6227-6235.

36. Comstock G.W. (1978). Tuberculosis in twins: a re-analysis of the prophet survey. Am. Rev. Respir. Dis, 117,621-624.

37. Davies, G.R., M.Pillay, A. W. Strumand D. Wilkinson (1999). Emergence of multidrug-resistant tuberculosis in a community - based directly observed treatment programme in rural South Africa. Int. J. Tuberc. Lung. Dis, 3, 799:804.

38. Davinder P. S. Dosanjh., Mustafa Bakir., Kerry A. Millington1, Ahmet Soysal, Yasemin Aslan, Serpil Efee, Jonathan J. Deeks, Ajit Lalvani (2011). Novel *M. t u b e r c u l o s i s*Antigen-Specific T-Cells are Early Markers of Infection and Disease Progression. Plos One, vol 6, 12, e28754.

39. Delgado, J. C., A. Baena, S. Thimand A.E. Goldfield (2002). Ethinic specific genetic association with pulmonary tuberculosis. J. Infect. Dis, 186, 1463-1468.

40. Delogu, G., C. Pusceddu, A. Bua, G. Fadda, M.J. Brennan and S. Zanetti (2004). *Rv1818c*-encoded PE_PGRS protein of *Mycobacterium tuberculosis* is surface exposed and influences bacterial cell structure. Mol. Microbiol, 52, 725-733.

41. Desprez, R.M. & C.R. Heim. (1990). *Mycobacterium tuberculosis*. In GL, Mandell R.F. Douglas Jr, J.E. Bennett (Eds), Principles and Practice of Infectious Diseases (3rded). New York: Churchill Livingstone, 1990, pp. 1877-1906.

42. Dietrich, J., C.V.Lundberg and P.Andersen (2006). TB vaccine strategies-what is needed to solve a complex problem? *Tuberculosi*, 86,163-168.

43. Enarson, D.A., H. L. Reider, T. Arnadottir and A. Trebucq (2000). Management of tuberculosis: a guide for low-income countries, 5thedn. Paris: International Union against Tuberculosis and Lung Disease pp.1-89.

44. Espitia, C., J. P. Laclette, M. Mondragon-Palomina, A. Amador, J. Campuzano *et al*. (1999). The PE-PGRS glycine-rich proteins of *Mycobacterium tuberculosis* fibronectin-binding proteins. Microbiol,145, 3487-3495.

45. Fine P.E. (2000).BCG vaccines and vaccination. In: Tuberculosis: a comprehensive international approach. Reichman LB, Hershfield ES, editors. New York: Marcel Dekker; p. 503-518.

46. Fine, P.E.M (1995). Vaccination in protection by BCG: implications of and for heterologous immunity. Lancet, 346, 1339-1345.

47. Foulds, J. and R.O'. Brien (1998). New tools for the diagnosis of tuberculosis: the respective of developing countries. Int. J. Tuberc. Lung. Dis, 2, 778-783.

48. Gennaro, M.L. (2000). Immunologic diagnosis of tuberculosis, *Clin. Infect. Dis.* 30 (Suppl. 3): S243-S246.

49. Gordon, S.V., R. Brosch, A. Billact, T.Garnier, K. Eiglmeier and S.T. Cole (1999). Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome assays. Mol. Microbiol, 32, 643-655.

50. Grange, J. (1996). Other mycobacterial diseases. In: Grange J ed. Mycobacteria and human disease. London, UK: Arnold, 188-203.

51. Greenwood, C.M., T.M. Fujiwara, L.J. Boothroyd, M.A. Miller, D. Frappier*et al*. (2000). Linkage of tuberculosis to chromosome 2q35 loci, including NRAMP1, in a large aboriginal Canadian family. Am. J. Hum Genet, 67, 405-416.

52. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena (2002). Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol, 20, 621-667.

53. Harboe,M., and H. G. Wiker (1992). The 38-kD protein of *Mycobacterium tuberculosis*: a review. *J. Infect. Dis.* 166: 874-884.

54. Harboe,M., T. Oettinger, H. G. Wiker, I. Rosenkrands, and P. Andersen(1996). Evidence for Occurrence of the ESAT-6 Protein in *Mycobacterium tuberculosis* and Virulent *Mycobacterium bovis* and for its Absence in *Mycobacteriumbovis* BCG. Infect. Immun, 64(1), 6-22.

55. Haslov, K., A. Andersen, S. Nagai, A. Gottschau, T. Sorensen *et al*. (1995). Guinea pig cellular immune responses to proteins secreted by *Mycobacterium tuberculosis*. *Infect. Immun.* 63: 804-810.

56. Henderson, R.A., S. C. Watkins, and J.L. Flynn (1997). Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. J. Immunol, 159, 635-643.

57. Hewinson, R.G., H. M. Vordermeier and B.M. Buddle (2003). Use of the bovine model of tuberculosis for the development of the improved vaccines and diagnostics. *Tuberculosis* (Edinburgh) 83: 119-130.

58. Hill, A.V. (1998). The immunogenetics of the human infectious diseases. Annu. Rev. Immunol, 16,593-617.

59. Hsu, T., S. M. Hingley-Wilson, B. Chen, M. Chen, A. Z. Dai, P.M. Morin, *et al*. (2003). The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl AcadSci U S A, 100, 12420-5.

60. Jasmer, R.M., P. Nahid and P.C. Hopewell (2002). Clinical practice. Latent tuberculosis infection. N. Eng. J. Med, 347, 1860-1866.

61. Joint Tuberculosis Committee of the British Thoracic Society (1998). Chemotherapy and management of tuberculosis in the United Kingdom: recommendations. Thorax, 53, 536-548.

62. Kulshrestha, A., A. Gupta, N. Verma, S.K. Sharma, A. K. Tyagi and V. K. Chaudhary (2005).Expression and purification of recombinant antigens of *Mycobacterium tuberculosis* for application in serodiagnosis. *Protein. Expr. Purif.* 44(1): 75-85.

63. L. Baassi., K. Sadki, F. Seghrouchni, S. Contini, W. Cherki, N. Nagelkerke,A. Benjouad, C. Saltini, V. Colizzi, R. El Aouad, M. Amicosante(2009). Evaluation of a multi-antigen test based on B-cell epitope peptides for the serodiagnosis of pulmonary tuberculosis. Int. J. Tuberc. Lung Dis. 13(7): 848–854.

64. Lalvani,A.,P.Nagvenkar,Z.Udwadia,A.A. Pathan, K.A. Wilkinson *et al*.,**(**2001). Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urbanIndians.J.Infect. Dis, 183, 469-477.

65. Lane, P.J.L. and T. Brocker (1999).Developmental regulation of dendritic cell function. Curr. Opinion Immunol, 11, 308-331.

66. Laqueyrerie, A., P. Militzer, F. Romain, K. Eiglmeier and S.T. Cole (1995).Cloning, sequencing, and expression of the *apa* gene coding for the *Mycobacterium tuberculosis* 45/47-kilodolton secreted antigen complex. *Infect. Immun.* 63: 4003-4010.

67. Lee, F. and R.S. Holzman (2002). Evolution and current use of the tuberculin test. Clin. Infect. Dis, 34, 365-370.

68. Lewis, K.N., R. Liao, K. M. Guinn, M.J. Hickey, S. Smith, M. A. Behr and D. R. Sherman (2003). Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacillus Calmette-Guerin attenuation. JInfect Dis, 187, 117-123.

69. Liu, X.Q., D. Dosanjh, H. Varia, K. Ewer, P. Cockle, *et al.* (2004). Evaluation of T-cell responses to novel RD1- and RD2-encoded *Mycobacterium tuberculosis* gene products for specific detection of human tuberculosis infection. *Infect. Immun.* 72 (5): 2574 -2581.

70. Lodes, M.J., D.C. Dillon, R. Mohamath, C.H. Day and D. R. Benson, L.D. Reynolds, P. McNeill, D.P. Sampaio, Y.A. Skeiky, R. Badaro, D.H. Persing, S.G. Reed and R.L. Houghton (2001). Serological expression cloning and immunological evaluation of MTB48, a novel *Mycobacterium tuberculosis* antigen. *J. Clin. Microbiol.* 39(7): 2485-2493.

71. Lu Zhang, Qingzhong Wang, Wenjie Wang, Yanyan Liu, Jie Wang, Jun Yue, Ying Xu, Wenxi Xu, ZhenLing Cui, Xuelian Zhang and Honghai Wang(2012). Identification of putative biomarkers for the serodiagnosis of drug-resistant *Mycobacterium tuberculosis*. Proteome Science, 10:12.

72. Mahairas, G.G., P.J. Sabo, M.J. Hickey, D.C. Singh and C. K. Stover (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J. Bacteriol. 178(5), 1274 -1282.

73. Målen H, Søfteland T, Wiker HG. (2008) Antigen analysis of Mycobacterium tuberculosis H37Rv culture filtrate proteins. *Scand J Immunol*.67 (3), 245-52.

74. Malik, SW., L. Abel, H. Tooker, A. Poon, L. Simkin*et al*. (2005). Alleles of the NRAMP-1 gene are risk factors for pediatric tuberculosis disease. Proc. Natl. Acad. Sci. USA 102, 12183-12188.

75. Manca, C., K. Lyashchenko, R. Colangeli and M. L. Gennaro (1997). MTC28, a novel 28-kilodalton proline-rich secreted antigen specific for the *Mycobacterium tuberculosis* complex. *Infect. Immun.* 65(12): 4951-4957.

76. Miller, N.S., S.G. Hernandez and T.J. Cleary (1994). Evaluation of Gen-probe amplified *Mycobacterium tuberculosis*Direct test and PCR for direct detection of *Mycobacterium tuberculosis* in clinical specimens. J. Clin. Microbiol, 32, 393-397.

77. Mogues,T., M. E. Goodrich, L. Ryan, R. Lacourse and R.J. North.(2001). The Relative importance of T cell subsets in immunity and immunopathology of airborne *Mycobacterium tuberculosis* infection in mice. J. Exp. Med, 193, 271-280.

78. Mukherjee P, Dutta M, Datta P, *et al.* (2006). The RD1-encoded antigen Rv3872 of *Mycobacterium tuberculosis* as a potential candidate for serodiagnosis of tuberculosis. Clin Microbiol Infect, 13: 146–152.

79. Nagai,S., H. G. Wiker, M. Harboe and M. Kinomoto (1991). Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*.Infect. Immun, 59, 372-382.

80. Noordhoek, G. T., A. H. Kolk, G.Bjune, D. Catty, J.W. Dale *et al*. (1994).Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind study among seven laboratories. J. Clin. Microbiol, 32, 277-284.

81. Nunn, P., D. Kibuga, S. Gathua, Brindle R, Imalingat A, Wasunna K, Lucas S, Gilks C, Omwega M, Were J, *et al*. (1991). Cutaneous hypersensitivity reactions due to thioacetazone in HIV-1 seropositive patients treated for tuberculosis. Lancet, 337, 627-630.

82. Oettinger, T., M. Jorgensen, A. Ladefoged, K. Haslov, and P. Andersen (1999). Development of *Mycobacterium bovis* BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. Tuber. Lung. Dis, 79, 243-250.

83. Okewara, A., C. Whalen, F. Byekwaso, M. Vjecha, T. Aisu, C. Day-Lally, J. Nakibali, K. Edmonds, S. Nyole, R. Huebner, J. Johnson, D. Hom, R. Wallis, A. Morrissey, T. Daniel, R. Mugerwa, J. Ellner (1994). Randomised trial of thioacetozone and rifampicin containing regimens for pulmonary tuberculosis in HIV infected Ugandans. Lancet, 344, 1323-1328.

84. Olsen, A.W., P.R. Hansen, A. Holm and P. Andersen (2000). Efficient protection against *Mycobacterium tuberculosis* by vaccination with a single subdominant epitope from the ESAT-6 antigen, Eur. J. Immunol, 30, 1724-1732.

85. PaiM., L. W. Riley and J. M. Colford Jr. (2004). Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. Lancet. Infect. Dis, 4, 761-776.

86. Pallen M.J. (2002).The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system? Trends Microbiol, 10(5), 209-212.

87. Pasqualoto, K.F.M. & Ferreira, E.I.(2001). An approach for the rational design of new antituberculosis agents. Curr. Drug. Targets, 2, 427-437.

88. Pathan, A., K. A. Wilkinson, P. Klenerman, H. McShane, R. N. Davidson, G. Pasvol, *et al*. (2001). Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in *Mycocbacterium tuberculosis*- infected individuals: associations with clinical disease state and effort of treatment. J. Immunol. 167,5217-5225.

89. Petrini, B. & Hoffner S. (1999). Drug-resistant and multidrug-resistant tubercle bacilli. Int. J. Antimicrob. Agents, 13, 93-97.

90. Pollock, J.M. and P. Andersen (1997). Predominant recognition of the ESAT-6 protein in the first phase of interferon with *Mycobacterium bovis* in cattle. *Infect. Immun.* 65: 2587-2592.

91. Pottumarthy,S., A. C. Harrison and V.C. Wells (1999). Evaluation of tuberculin gamma interferon assay: potential to replace the montoux skin test. J.Clin.Microbiol, 37, 3229-3232.

92. Pym, A.S., P. Brodin, R. Brosch, M. Huerreand S.T. Cole (2002). Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. Mol. Microbiol, 46, 709-717.

93. Pym, A.S., P. Brodin, L. Majlessi, R. Brosch, C. Demangel, *et al*. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat. Med, 9, 533-539.

94. Rachman, H., M. Strong, U. Schaible, J. Schuchhardt, K. Hagens*et al*. (2006). *Mycobacterium tuberculosis* gene expression profiling within the context of protein networks. Microbes and Infection, 8,747-757.

95. Randhawa, P.S. (1990). Lymphocyte subsets in granulomas of human tuberculosis: an in situ immune fluorescence study using monoclonal antibodies. Pathology,22**,** 153-155.

96. Ravn, P., A. Demissie, T. Eguale, H. Wondwosson, D. Lein, H. A. Amoudy, A.S. Mustafa, A. K. Jensen, A. Holm, I. Rosenkrands, F. Oftung, J. Olobo, F. Reyn and P. Andersen (1999). Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis. J. Infect. Dis.* 179: 637-6450.

97. Rengarajan, J., B.R. Bloom and E.J. Rubin.(2005).Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. Proc. Natl. Acad. Sci. U.S.A, 102, 8327-8332.

98. Renshaw, P.S., P. Panagiotidou, A. Whelan, S.V. Gordon, R.G. Hewinson, R.A. Williamson, M.D. Carr (2002). Conclusive evidence that the major T-cell antigens of the *Mycobacterium tuberculosis* complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10 and the ESAT-6.CFP-10 complex: implications for pathogenesis and virulence. J BiolChem, 277, 21598-21603.

99. Scarpellini P., S. Tasca, L. Galli, A. Beretta, A. Lazzarinand C. Fortis (2004). Selected pool of peptides from ESAT-6 and CFP-10 proteins for detection of Mycobacterium tuberculosis infection, J ClinMicrobiol, 42(8), 3469-3474.

100. Schaible, U.E., K. Hagens, K. Fischer, H.L. Collins and S. H. Kaufmann (2000). Intersection of group-I CD1 molecules and mycobacteria in different intracellular compartments of dendritic cells. J. Immunol.164, 4843-4852.Small, P.M. & Fujiwara, P.I (2001). Management of Tuberculosis in the United States. N. Engl .J. Med., 345, 189-200. Skjøt, R.L., T. Ottinger, I. Rosengrands, P. Ravn, I. Brock, *et al* (2000).Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens, Infect. Immun, 68(1), 214-220.

101. Stead, W.W. (1992). Genetic and resistance to tuberculosis. Am. Intern. Med, 116, 937-941.

102. Sturum, A.W. (2006). The UKZN "Outbreak". Expert consultation on drug-resistant tuberculosis. Johensberg: MRC Unit for TB Epidemiology and Interventions Branch.

103. Tekaia,F., S. V. Gordon, T. Garnier, R. Brosch, B. G. Barrel and S. T. Cole (1999). Analysis of the proteome of *Mycobacterium tuberculosis* in silico. Tubercul. Lung. Dis, 79, 329-342.

104. Tuberculosis Research Centre (ICMR), Chennai (1999) Fifteen year follow up of trial of BCG vaccines in south India for tuberculosis prevention. Indian J Med Res, 110, pp 56-69.

105. Turcotte, R. (1975). The participation of common and species-"specific" antigens of mycobacteria in the tuberculin skin reaction. Can. J. Microbiol, 21, 774-783.

106. Van Pittius, N.C.G., J. Gamieldien, W. Hide, G.D. Brown, R.J. Siezen*et al*. (2001). The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C gram positive bacteria. Genome Biology, 2(10), 0044.1-0044.18.

107. Wards, B.J., G.W. de Lisle and D.M. Collins (2000). An esat-6 knockout mutant of *Mycobacterium bovis* produced by homologous recombination will contribute to the development of a liver tuberculosis vaccine. Tuber. Lung. Dis. 80, 185-189.

108. WHO (2003). Global tuberculosis Programme. Treatment of tuberculosis: guidelines for national programmes, 3rd edn. (WHO/CDS/TB/2003.13) Geneva.

109. WHO report (2000) Joint tuberculosis programme review in India. New Delhi, India: (WHO/TB/97,231).

110. WHO vaccine preventable disease (2000) Global summary.

111. WHO (2002). Global Tuberculosis Control, Surveillance, Planning, Financing: WHO/CDS/ Tuberculosis/2002. 295.

112. World Health Organization (2011). Global Tuberculosis Control: Surveillance, planning financing. WHO report. WHO/HTM/TB/2011.

113. World Health Organization (2012). Global Tuberculosis Control: Surveillance, planning financing. WHO report. WHO/HTM/TB/2012.6

114. Xu Jia-Nan, Jian-Ping Chen and Da-Li Chen (2012). Serodiagnosis Efficacy and Immunogenicity of the Fusion Protein of *Mycobacterium tuberculosis* composed of the 10-Kilodalton Culture Filtrate Protein, ESAT-6, and the Extracellular Domain Fragment of PPE68. Clin. Vaccine Immunol. 19(4): 536.

115. Zou, Y.L., J.D. Zhang, M. H. Chen, G.Q. Shi, J. Prignot *et al*. (1994).Serological analysis of pulmonary and extrapulmonary tuberculosis with enzyme-linked immunosorbent assays for anti-60 immunoglobulins. Clin. Infect. Dis, 19, 1084-1091.